Regulatory enzymes of mitochondrial B-oxidation as targets for treatment of the metabolic syndrome

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CHAPTER 5

INHIBITION OF CARNITINE PALMITOYLTRANSFERASE 2 BY AMINOCARNITINE PREVENTS KETOACIDOSIS IN A MOUSE MODEL OF TYPE I DIABETES: A METABOLOMIC APPROACH

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ABSTRACT

Inhibition of mitochondrial fatty acid β-oxidation (FAO) has been proposed for prevention of hyperglycemia and ketoacidosis in patients with type I diabetes. We have used a metabolomic approach to characterize the acute effects of FAO inhibition with the carnitine palmitoyltransferase 2 (CPT2) inhibitor L-aminocarnitine (L-AC) in mice. Type I diabetes with severe hyperglycemia and ketoacidosis was induced using alloxan in male C57Bl6/OlaHsd mice. Diabetic and control mice were treated with L-AC (16 mg/kg) and sacrificed after 6 hours.L-AC treatment of control and diabetic mice reduced plasma ketone bodies in plasma, resolving the ketonemia. Blood glucose levels were lowered only in the control mice. Long-chain acylcarnitines in L-AC treated animals as well as in control animals were highly elevated in liver, associated with a marked hepatic steatosis. Remarkably, L-AC strongly reduced branched chain amino acids in diabetic mice, indicative for less breakdown of skeletal muscle upon L-AC treatment.

In conclusion, acute CPT2 inhibition by L-AC improves hyperketonemia in type I diabetes, however, for chronic therapeutic treatment L-AC may not be feasible because it causes hepatic steatosis.

INTRODUCTION

In patients with type I diabetes, diabetic ketoacidosis (DKA) is an acute life-threatening event comprising three components: hyperglycemia, ketonemia and metabolic acidosis. The underlying cause is a hormonal disbalance predisposing towards a catabolic state in which insulin is almost absent and elevated levels of counter-regulatory hormones like glucagon, catecholamines and glucocorticoids are noted. In this situation, a sequence of events is initiated by diminished glucose uptake by peripheral tissues and induced lipolysis in adipose tissue. In liver, the increase of free fatty acids, together with an increased activity of enzymes involved in fatty acid b-oxidation (FAO) and ketogenesis, results in an enhanced production of ketone bodies. Elevated concentrations of counter-regulatory hormones induce proteolysis which, together with high concentrations of glycerol and lactate, results in the delivery of large amounts of gluconeogenic precursors to the liver and kidney. Combined with high activity of gluconeogenic enzymes, a high rate of gluconeogenesis can be anticipated that contributes to hyperglycemia [1]. Causes of DKA are infections and omission as well as undertreatment with insulin. Particularly, children and adolescents with type I diabetes appear to be more susceptible to DKA, likely as a reflection of their often irregular feeding pattern and less controlled insulin therapy [2–5]. Prevention of DKA requires a strict insulin treatment regime. So far, effective therapy for treatment of ketoacidotic shocks depends on insulin administration and intensive hydration.

In healthy conditions, ketogenesis takes place in the liver as a response to fasting or prolonged exercise to provide organs like the brain and heart with sufficient energy. Ketone bodies (acetoacetate and β-hydroxybutyrate) are produced from acetyl-CoA, the product of FAO [6,7]. Since ketogenesis is localized in liver, interference with hepatic FAO has been proposed as a therapeutic option to curtail DKA. To this purpose, several FAO inhibitors have been developed. These inhibitors target the so-called carnitine shuttle, which functions in the import of acyl-CoAs into the mitochondrial matrix, the initial step of the FAO pathway. Most of these compounds are modified long-chain fatty acids, such as the acyl-substituted 2-oxirane carboxylic acids etomoxir and 2-tetradecylglycidic acid (TDGA). The specific target of these drugs is carnitine palmitoyltransferase 1 (CPT1) that converts acyl-CoAs into acyl-carnitines in the first step of the carnitine shuttle. Etomoxir and TDGA have been tested clinically, however, severe side effects such as hypertrophic cardiomyopathy and hepatic steatosis has prevented their introduction into daily clinical practice [8–10]. To the best of our knowledge these compounds have never been tested in animal models or humans with insulin-dependent diabetes. Besides being inhibitors of FAO, these drugs are also direct ligands of PPARα, a nuclear hormone receptor and a well-known inducer of FAO and ketogenesis upon fasting [11–13]. Another class of compounds, which inhibit FAO, are substituted carnitines such as L-3-amino-4-trimethylaminobutyric acid (L-aminocarnitine (L-AC)). L-AC acts mainly on CPT2 [12,14,15], the inner-mitochondrial carnitine palmitoyltransferase that catalyzes the

reverse reaction of CPT1. In vitro data suggest that L-AC may also inhibit CPT1 at higher concentration [12,16]. L-AC is a potent inhibitor of FAO in vivo in rodents [17]. Importantly, recent data indicate that L-AC is not a direct PPARα agonist, in contrast to above mentioned CPT1 inhibitors [12]. Yet, no data are available on the efficacy of L-AC in the treatment of DKA in a model of type I diabetes.

We studied the metabolic consequences of FAO inhibition by L-AC in alloxan-induced type I diabetes in mice. Moreover, we examined the effects of this compound on expression levels of genes involved in glucose metabolism, hepatic FAO and ketogenesis.

MATERIAL AND METHODS

Animals

Male C57Bl6/OlaHsd mice were treated with alloxan (i.v. 80 mg/kg in PBS) to induce type I diabetes. Another group of mice was injected with vehicle (PBS) to serve as control group. Blood glucose levels were measured two days after treatment by tail bleeding. A blood glucose value exceeding 18 mmol/l was considered indicative for successful induction of diabetes. One week after alloxan or vehicle treatment, mice were i.p. injected with either aminocarnitine (L-AC, a gift from Sigma-Tau) or vehicle (PBS) in a total volume of 200 µl at a dose of 16mg/kg. Six hours after L-AC or vehicle injection, mice were sacrificed and blood and tissues were collected for assessment of biochemical parameters. During the 6 hour experiment, blood glucose levels were measured at the start of the experiment and subsequently every three hours (One Touch glucose meter, LifeScan Benelux, Beerse, Belguim). During the time course of the experiment, mice had free access to water, but were deprived from food.

Tissue biochemical metabolic parameters

Plasma triglycerides (TG), total cholesterol and total fatty acids were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany, Wako Chemicals, Neuss). Total ketone bodies were determined with the total ketone body kit (Wako Chemicals, Neuss, Germany). Lactate was measured using standard laboratory methods. Acylcarnitines were determined according to the method of Chase et al. [18] as described by Derks et al. [19]. Liver lipid extraction was performed according to the procedure of Bligh and Dyer [20]. Protein concentrations of these livers were determined according to the Lowry method [21]. Hepatic concentrations of TG, free and total cholesterol were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany and Wako Chemicals, Neuss, Germany). The hepatic glycogen levels were measured as described by Keppler [22]. Malonyl-CoA content of the liver was measured according to the protocol of Demoz et al. [23] with minor modifications on a Nova-Pak Reversed Phase C-18 column (Waters, Etten Leur, The Netherlands). The fatty acid profile was determined according to the

method of Gates et al.[24]. Amino acids were quantified by cation exchange chromatography. Column material consists of Ultrapac 8 remaining resin as delivered by manufacturer. Analysis was performed on a Biochrom20® amino acid analyzer according to manufacturer's protocol. Measurements of TCA cycle metabolites were performed according to the method of Gates et al.[24] modified for tissue homogenates. Tissue homogenates of 15% (w/v; using about 250 mg tissue) in PBS were sonicated and centrifuged. Supernatants were extracted with an equivolume of ether: ethylacetate (1:1, v:v). The organic layers were collected, evaporated and the residues were derivatized using BSTFA: TCMS: pyridine (5:0.06:1, v:v:v). Samples were injected split and splitless on a Finnigan Trace Plus GCMS (Interscience, Breda, The Netherlands). Metabolites were separated with a Grace AT™-1701 column (Grace Davison Discovery Science), Length 30 meters, ID 0.25 mm, Film 0.25µm. Temperature program: 5 min at 80 degrees C followed by a program rate of 4°C/min. until 280°C. Separated TCA cycle components were identified and quantified on the MS analyzer in Electron Impact mode.

RNA isolation and mRNA levels by Real Time PCR

Liver was treated with TRIzol (Invitrogen, Paisley, UK) according to manufacturer's guidelines to isolate RNA. The concentration of RNA was determined using the Nanodrop system (ND1000, Isogen Life Science, IJsselstein, The Netherlands). The cDNA reaction was performed using 1 µg of RNA per sample according to manufacturer's protocol using M-MLV-Reverse Transcriptase (Sigma, Zwijndrecht, The Netherlands). Quantitative real time PCR was performed using the Applied Biosystems 7900HT system with the FAST taqman PCR protocol (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). PCR results were normalized to 36B4 mRNA levels.

Statistics

Statistics were performed using non-parametric Kruskall-Wallis, followed by Mann-Whitney U test corrected for multiple testing. A p-value of $p<0.05$ was defined to be significant different. Calculations were made in SPSS 14.0 for Windows (SpSS, Chicago, IL, USA). All values are represented as mean ± sd.

RESULTS

Treatment of diabetic mice with L-AC prevents hyperketonemia

Mice treated with a single i.v. injection of alloxan showed major hyperglycemia with values >18 mmol/l. These mice displayed polyurea as well as hyperphagia and polydipsia as indication of severe diabetes. Body weight of diabetic mice decreased during the 7 days of hyperglycemia (24.9 ± 1.3 g at time of alloxan injection ver-

Table 1: *Plasma concentrations of various metabolites in mice at 6 hours after injection with either vehicle or 16 mg/kg L-AC.* Values are represented as mean ± sd (n=6-8). * p<0.05 vs control vehicle, # p<0.05 vs alloxan vehicle.

Plasma parameters	control	control + L-AC	alloxan	alloxan + L-AC
Triglycerides (mmol/l)	0.7 ± 0.2	1.4 ± 0.8	1.6 ± 1.2*	2.3 ± 1.7
Tot cholesterol (mmol/l)	3.0 ± 0.3	2.8 ± 0.4	3.3 ± 0.6	3.1 ± 0.7
Free fatty acids (mmol/l)	0.4 ± 0.0	1.2 ± 0.4*	0.5 ± 0.3	1.0 ± 0.3#
Lactate (mmol/l)	6.3 ± 1.2	4.7 ± 0.9	6.5 ± 1.5	7.0 ± 1.8

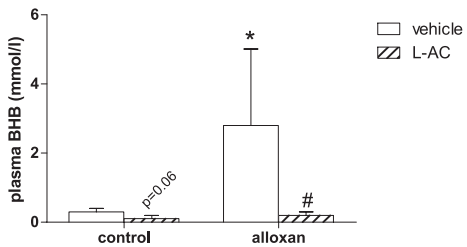

Figure 1: ***Plasma BHB levels of the four groups of mice after 6 hours of treatment with either vehicle or 16 mg/kg L-AC.*** Values are represented as mean ± sd (n=6-8). * p<0.05 vs control vehicle.

sus 21.9 ± 1.9 g at start of the treatment). Sham-treatment with PBS did not affect these parameters. Plasma free fatty acid concentration was not affected by alloxan treatment (Table 1). Plasma β-hydroxybutyrate (BHB) levels in diabetic mice, however, were significantly higher compared to non-diabetic control mice (Figure 1: +800%; p<0.05). A single i.p. injection of L-AC normalized BHB levels in diabetic mice. Moreover, the decrease in BHB levels were more pronounced in diabetic mice (Figure 1: -93%; p<0.05) compared to healthy controls (Figure 1: -66%; p=0.06). Free fatty acid levels were more than doubled after treatment with a single dose of L-AC in both control and diabetic mice (Table 1). Interestingly, glucose levels were only significantly affected in control mice treated with L-AC. L-AC did not lower blood glucose in diabetic mice (Figure 2). Plasma lactate and cholesterol levels were not altered by either alloxan or treatment with L-AC (Table 1) in either diabetic or non-diabetic mice. Plasma TG levels were higher in diabetic mice as compared to healthy control mice (Table 1). Treatment with L-AC did not have additional effects on plasma TG, although there was a tendency towards an increase upon L-AC treatment in both groups (Table 1). Thus, L-AC is able to prevent hyperketonemia in mice with alloxan-induced diabetes.

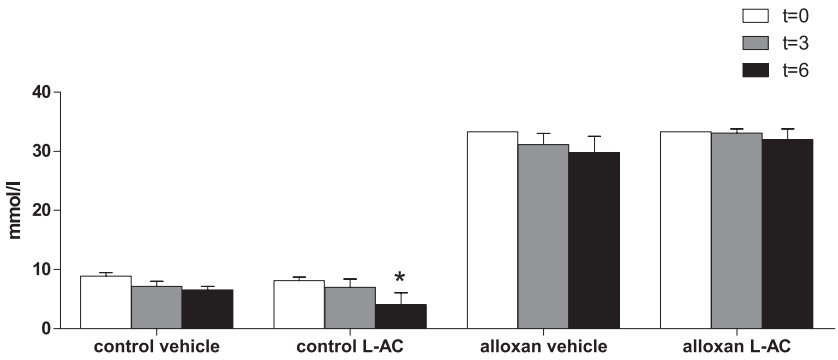

Figure 2: ***Glucose values of the four groups of mice during the 6 hours of treatment with either vehicle or 16 mg/kg L-AC.*** Values are represented as mean ± sd (n=6-8). * p<0.05 vs control vehicle. Due to the upper range of the glucose meter of 33 mmol/l, values above 33 mmol/l could not be measured in this experiment. Therefore, time point 0 of the alloxan animals has been set to 33mmol/l and has no sd bar.

L-AC strongly affects hepatic metabolism

The liver produces glucose and ketone bodies, which serve as energy substrates for other organs during fasting. We looked at changes in hepatic intermediate metabolism to profile the effects of L-AC treatment on alloxan-induced diabetes. Hepatic glycogen levels showed a remarkable decrease in non-diabetic control mice after short-term treatment with L-AC (Table 2: -40%; p<0.05). However, no significant effect of L-AC on hepatic glycogen content was observed in the alloxan-treated mice

Table 2: *Hepatic metabolic parameters of mice after 6 hours of treatment with either vehicle or 16 mg/kg L-AC.* **Values are represented as mean ± sd (n=6-8). * p<0.05 vs control vehicle, # p<0.05 vs alloxan vehicle.**

Hepatic parameters	control	control + L-AC	alloxan	alloxan + L-AC
Protein (mg/g liver)	137.2 ± 15.3	121.3 ± 31.5	150.0 ± 31.7	138.0 ± 25.6
Triglycerides (umol/g liver)	18.4 ± 3.6	34.8 ± 4.6*	5.9 ± 3.4*	25.9 ± 9.3#
Tot cholesterol (umol/g liver)	10.4 ± 0.8	12.4 ± 2.0*	11.3 ± 1.7	10.9 ± 0.8
Free cholesterol (umol/g liver)	6.4 ± 0.4	8.4 ± 1.6*	7.6 ± 1.5	10.9 ± 0.8#
Glycogen (umol/g liver)	350 ± 107	212 ± 50*	356 ± 140	294 ± 116
Malonyl-CoA (nmol/g liver)	11.1 ± 3.5	10.7 ± 1.7	20.8 ± 10.2	14.5 ± 6.6

(Table 2). The results on hepatic glycogen mirror the effects observed on blood glucose levels.

Defects in FAO are routinely detected in acylcarnitine spectrum analysis. In order to determine the effect of L-AC treatment, we determined the hepatic acylcarnitine profile. Alloxan treatment had only a minor effect on acylcarnitine profiles, although the concentration of free carnitine was almost doubled (Table 3). L-AC treatment caused major changes in the long-chain acylcarnitine profiles (Figure 3). In both healthy control and diabetic mice, treatment with L-AC caused a significant increase in long-chain acylcarnitine (total of C16:0; C18:1; C18:2 and C18:0 carnitine) levels in liver (Figure 3: ~32 times for treated control mice and ~11 times for treated diabetic mice, respectively). Hepatic TG levels were reduced in diabetic mice compared to control mice (Table 2: -68%; $p<0.05$). Upon inhibition of FAO by L-AC hepatic TG levels were significantly increased in the control (Table 2: +190%; $p<0.05$), but more pronounced in diabetic mice (Table 2: +440%; $p<0.05$). Combined with the acylcarnitine results, these results are consistent with inhibition of FAO.

Table 3: *Free carnitine levels of mice after 6 hours of treatment with either vehicle or 16 mg/kg L-AC.*
Values are represented as mean ± sd (n=6-8). * p<0.05 vs control vehicle, # p<0.05 vs alloxan vehicle.

Free carnitine levels (nmol/g)	control	control + L-AC	alloxan	alloxan + L-AC
liver	164.4 ± 25.5	332.0 ± 38.5*	309.5 ± 97.1*	345.7 ± 61.4
muscle	117.9 ± 20.6	113.8 ± 22.6	160.7 ± 60.8	144.3 ± 44.2

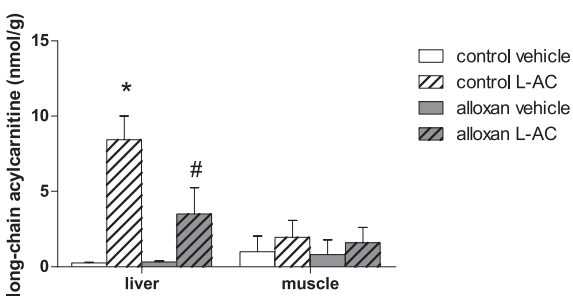

Figure 3: ***Long-chain acylcarnitine (sum of C16:0, C18:2, C18:1 and C18:0) accumulation in liver and skeletal muscle.*** Values are represented as mean ± sd (n=6-8). * p<0.05 vs control vehicle, # p<0.05 vs alloxan vehicle.

We measured hepatic malonyl-CoA levels, which are an important regulator of FAO flux. Remarkably, despite high ketone body synthesis, basal malonyl-CoA levels only tended to be elevated in the diabetic animals, suggesting repressed FAO flux. L-AC treatment did not affect hepatic malonyl-CoA levels in control or diabetic mice (Table 2).

An enhanced activity of FAO results in an increased production of acetyl-CoA. In the liver, formation of ketone bodies is an important route to metabolize acetyl-CoA, but acetyl-CoA is also used in the TCA cycle for the generation of energy. We determined hepatic content of intermediates of the TCA cycle to trace the effects of insulin deficiency in the absence and presence of L-AC. Citrate, succinate, fumarate and malate levels were all increased in livers of diabetic mice. Treatment with L-AC lowered hepatic fumarate and malate levels. For hepatic citrate and succinate values, a tendency towards lower levels was observed, which did not reach statistical significance (Table 4). Our results indicate that in alloxan-induced diabetes TCA cycle

Table 4: *TCA metabolites of mice after 6 hours of treatment with either vehicle or 16 mg/kg L-AC.* **Values are represented as mean ± sd (n=6-8). * p<0.05 vs control vehicle, # p<0.05 vs alloxan vehicle.**

	control	control + L-AC	alloxan	alloxan + L-AC
TCA metabolites liver				
Citrate (nmol/g)	1265 ± 598	851 ± 705	5852 ± 6121*	3154 ± 2886
Malate (nmol/g)	6584 ± 2567	3130 ± 113*	8659 ± 2408	4513 ± 781#
Succinate (nmol/g)	852 ± 211	712 ± 187	2211 ± 1646*	1201 ± 1058
Fumarate (nmol/g)	2451 ± 941	1117 ± 400*	3065 ± 733	1643 ± 332#
Succinate:fumarate	0.40 ± 0.19	0.69 ± 0.21	0.52 ± 0.26	0.52 ± 0.28
muscle				
Citrate (nmol/g)	243 ± 70	278 ± 105	448 ± 113*	343 ± 77
Malate (nmol/g)	1222 ± 109	1106 ± 208	1495 ± 482	855 ± 219#
Succinate (nmol/g)	407 ± 264	363 ± 213	264 ± 152	312 ± 183
Fumarate (nmol/g)	394 ± 159	422 ± 145	546 ± 219	404 ± 150
Succinate:fumarate	0.98 ± 0.51	0.75 ± 0.35	0.46 ± 0.14	0.70 ± 0.28

intermediates are increased (anaplerosis), whereas L-AC treatment leads to depletion of TCA cycle intermediates. Thus, metabolite analysis suggests that L-AC strongly affects hepatic metabolism.

We also measured acylcarnitines and TCA cycle intermediates in skeletal muscle. Free carnitine as well as long-chain acylcarnitine levels were not affected by diabetes and L-AC treatment. For TCA cycle metabolites, only citrate was significantly elevated in alloxan-induced diabetes. Treatment with L-AC only lowered malate levels in the diabetic animals (Table 4). Interestingly, diabetic mice showed a 50% lower ratio in the succinate:fumarate ratio compared to control mice. Treatment with L-AC increased this ratio by 60% in diabetic animals, normalizing it again. Thus, in contrast to the liver, L-AC caused only subtle changes in muscle metabolite content.

Changes in hepatic gene expression levels upon treatment with L-AC

To define the underlying mechanisms of the major changes in hepatic metabolism after induction of diabetes and L-AC treatment, we measured hepatic gene expression levels of genes involved in glucose metabolism, FAO, ketogenesis and lipogenesis. Gck (glucokinase) levels were lower in mice treated with alloxan, consistent with its regulation by insulin. L-AC treatment did not change expression of this gene in both groups (Figure 4). Expression levels of G6ph (glucose-6-phosphatase hydrolase) were increased upon L-AC treatment in non-diabetic mice, a response that was absent in the diabetic mice. For G6pt (glucose-6-phosphatase translocase), expression levels were increased in diabetic animals compared to non-diabetic mice. L-AC treatment normalized expression levels in the diabetic animals. Expression of Pepck was increased only in alloxan treated animals, indicative for increased gluconeogenesis. Subsequent treatment with L-AC decreased expression of Pepck. Expression levels of Pdk4 (pyruvate dehydrogenase kinase 4), which inactivates pyruvate dehydrogenase by phosphorylation, were massively increased in both control and diabetic mice after short-term treatment with L-AC (Figure 4).

Next, we analyzed expression of genes involved in FAO. In animal models of type I diabetes, lower hepatic CPT1a expression levels have been reported [25,26]. However, in our experiments Cpt1a mRNA expression levels were not altered in alloxan-induced diabetic mice. L-AC treatment significantly induced Cpt1a mRNA expression levels in both control and diabetic mice (Figure 4). Expression of Aox (acyl-CoA oxidase 1), Octn2 (organic cation/carnitine transporter) and Acc2 (acetyl-CoA carboxylase 2) was also induced by L-AC (Figure 4). In general this induction was somewhat weaker in the diabetic animals. Aox is an enzyme of peroxisomal β-oxidation. Octn2 is the Na+-dependent carnitine transporter that is involved in the uptake of carnitine in tissues. Acc2 synthesizes malonyl-CoA. Quite surprisingly, despite massive ketonemia no effects were observed on the expression of mitochondrial HMG-CoA synthase (mHMGCoAs) in diabetic animals.

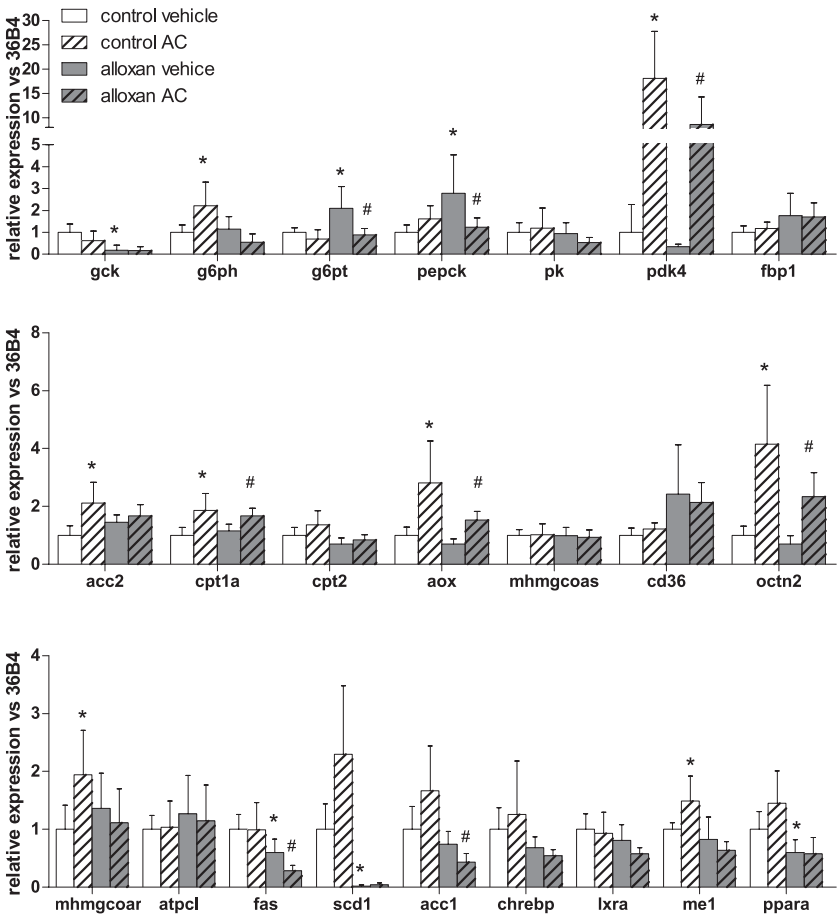

Figure 4: ***Hepatic gene expression levels of genes involved in FAO, glucose and lipid metabolism.*** Values are represented as mean ± sd (n=6-8). * $p<0.05$ vs control vehicle,

# $p<0.05$ vs alloxan vehicle.

Genes involved in lipogenesis like Acc1 (acetyl-CoA carboxylase) and Fas (fatty acid synthase) were significantly decreased in diabetic mice. Scd1 (stearyl-CoA desaturase) was almost undetectable in these animals. Treatment with L-AC lowered gene expression levels of Acc1 and Fas in diabetic mice even further. L-AC treatment did not influence gene expression levels of these lipogenic genes in control mice (Figure 4). Thus L-AC treatment has prominent effects on the gene expression of genes involved in FAO, an effect most probably mediated via PPARa.

Proteolysis was induced by alloxan treatment and partially normalized upon L-AC treatment

In alloxan-induced diabetic mice, plasma levels of branched-chain amino acids (leucine, isoleucine and valine) were elevated indicating breakdown of muscle protein. In non-diabetic control mice, L-AC did not alter branched chain amino acid concentrations in plasma. Treatment of diabetic mice with L-AC lowered branched chain amino acid concentrations by about 25%. Alanine levels were decreased by ~50% in the non-diabetic control animals. This effect was not observed in the diabetic mice (Table 5).

Table 5: *Branched-chain amino acids and other relevant amino acids of mice after 6 hours of treatment with either vehicle or 16 mg/kg L-AC.* Values are represented as mean ± sd (n=6-8). * p<0.05 vs control vehicle, # p<0.05 vs alloxan vehicle.

amino acids plasma	control	control +L-AC	alloxan	alloxan + L-AC
valine (umol/l)	170 ± 2	205 ± 60	580 ± 166*	418 ± 79
leucine (umol/l)	139 ± 13	163 ± 46	443 ± 127*	280 ± 52#
isoleucine (umol/l)	81 ± 6	88 ± 18	252 ± 64*	164 ± 28#
alanine (umol/l)	309 ± 70	148 ± 64*	298 ± 54	298 ± 115

DISCUSSION

The objective of this study was to elucidate whether inhibition of FAO using L-AC could contribute to a therapy for the treatment of DKA in type I diabetes. In patients suffering from type I diabetes insulin therapy is the first choice to treat hyperglycemia and ketoacidosis. However, in acute (life-threatening) DKA, an additional therapy like L-AC might be beneficial to rapidly reduce ketone body levels. We show in this study that L-AC rapidly decreases plasma BHB levels in diabetic mice. In control mice, blood glucose levels were reduced also upon L-AC treatment, however, this effect was not seen in diabetic mice. The main mechanism of action of L-AC was inhibition of FAO, as evidenced by an increase of long-chain acylcarnitines in liver (Figure 3) and heart (data not shown). This proves that L-AC inhibits CPT2 in vivo in both healthy and diabetic mice. As a consequence of the FAO inhibition, acute L-AC treatment caused hepatic steatosis. Although this is an unwanted side effect, we believe that the accumulated TGs will disappear after discontinuation of the L-AC treatment. Long term treatment with L-AC, however, may not be possible, since chronic steatosis might induce hepatic insulin resistance, atherosclerosis and other (cardiovascular) problems. In addition, we also tested a higher dose of L-AC

(32 mg/kg of body weight, unpublished results), which resulted in severe side effects and lethargy in the diabetic animals. We speculate that in the absence of insulin, major organs are not able to use glucose efficiently. Consequently, inhibition of FAO becomes live-threatening under these conditions. This indicates that L-AC or other FAO inhibitors may be used in the treatment of DKA, but probably additional therapy of insulin is required.

This is further illustrated by our finding that treatment of diabetic mice with L-AC caused a fast reduction of plasma BHB levels in these mice, but no reduction of blood glucose levels, which is in contrast to the control animals. This is a clear indication that insulin is needed to use glucose efficiently. In another study by our group, we found similar effects of whole body FAO inhibition on both blood glucose levels as well as plasma BHB levels. In these experiments, TDGA was used to block CPT1a, resulting in lower blood glucose levels as well as lower plasma BHB levels. Plasma values of FFA and TG were elevated in the TDGA study similar to what was found in the present study [27,28]. At the molecular level, the effects of insulin deficiency are reflected by the lowered expression levels of Gck and Scd1, which are known to be insulin-dependent genes [29–31].

Our finding that L-AC vey efficiently decreases plasma BHB, clearly shows that acetyl-CoA derived from FAO is used for the excessive ketogenesis in the diabetic animals. The lower hepatic TG levels in the diabetic animals may indicate also that fatty acids are preferentially oxidized. The insulin deficiency and the relative excess of glucagon mediate these effect, but the exact mechanism is unclear. Interestingly, hepatic gene expression levels of Cpt1a and mHMGCoAs, the rate-limiting enzymes in FAO and ketogenesis [32] were not different between healthy control mice compared to the diabetic mice. Moreover, we did not observe major changes in malonyl-CoA levels. This suggests that the acetyl-CoA synthesis rate and the partitioning of acetyl-CoA to ketogenesis are not determined by transcriptional mechanisms, but most likely by regulation of enzyme activity by other mechanisms.

An important difference between our current study and the TDGA study is the fact that TDGA is a direct PPARα agonist, whereas L-AC is not. L-AC was not able to act as a PPARα ligand in a recent in vitro study using expression analysis and luciferase reporter assays [12]. However, in contrast to the effects of L-AC observed in cell cultures, in vivo L-AC administration resulted in a strong activation of Pparα as evidenced by the induction of several PPARα target genes such as PDK4, CPT1a and Aox. We believe that this effect is due to the accumulation of fatty acids secondary to the inhibition of CPT2. As fatty acids are agonists of Pparα, this will result in increased expression levels of PPARα target genes. Moreover our results suggests that the increased levels of PPARα target genes after administration of TDGA may be a combined effect of PPARα activation by TDGA itself and the accumulating fatty acids.

A remarkable finding in our experiments was the observation that, in diabetic mice, plasma branched chain amino acids were not affected upon L-AC treatment whereas these levels were strongly elevated upon alloxan-induced diabetes alone. This is a clear indication that upon induction of diabetes by alloxan, protein degradation of the muscle is used to gather sufficient substrates for gluconeogenesis. The increase in hepatic TCA cycle intermediates may be a reflection of this phenomenon. Although the elevated protein degradation may be caused by low insulin and elevated counter regulatory hormones, it is unclear how treatment with L-AC in the diabetic animals protects against protein degradation. The protective effect of L-AC was noted only in the diabetic animals and not in the control mice.

In summary, we demonstrated that L-AC can be used for lowering plasma BHB levels in mice with type I diabetes. Our results also indicate that insulin may be crucial for basal glucose use. We speculate that FAO inhibitors might be used for treatment of DKA only in combination with insulin therapy. Chronic treatment of type I diabetic patients is counter indicated by the occurrence of hepatic steatosis.

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